

Full Length Research Paper

A marine bacterium, *Oceanobacillus* sp. Pinky, isolated from Algoa Bay sediment produces a thermostable glycoprotein flocculant

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Accepted 28 March, 2013

In this study, we report on the bioflocculant production potential of an *Oceanobacillus* sp. isolated from the marine sediments of Algoa Bay. The bacteria produced an extracellular bioflocculant optimally in the presence of sodium carbonate as source of carbon with flocculating activity of about 95.5%. Other optimal culture conditions included: tryptone as nitrogen source (flocculating activity, 84.5%); presence of Ca²⁺; inoculum size of 2% (v/v) and alkaline pH (10). Composition analyses revealed the purified bioflocculant to be thermostable and composed of polysaccharide and protein. Optimum dose of the purified bioflocculant for the clarification of 4 g/L kaolin clay suspension at neutral pH was 0.2 mg/ml. Scanning electron microscopic (SEM) observations were indicative of a porous bioflocculant structure while Fourier transform infrared (FTIR) spectrum revealed the presence of carboxyl, hydroxyl and amine groups. We proposed that the bacteria hold promise as a potential source of new bioflocculant compound(s).

Key words: *Oceanobacillus* sp., bioflocculant, glycoprotein, marine sediment, Algoa Bay.

INTRODUCTION

Flocculating agents are extensively used for the sedimentation of colloidal and cellular materials and thus applied in a wide range of industrial fields, including purification of drinking water, wastewater treatment, food industries, dredging and fermentation processes (Gao et al., 2006; Wang et al., 2010). Such flocculants could be inorganic or synthetic organic chemicals. Unfortunately, most of them have been found to be harmful to humans and the environment and as such their usages carry sig-

nificant risks (Sharma et al., 2006).

In recent years, marine microorganisms have been investigated as potential sources of bioflocculants. Typical examples include exopolysaccharides produced by marine microorganisms such as *Zoogloea* sp., *Pseudomonas* sp. and *Alteromonas maleolii* (Yim et al., 2007). According to Zhang et al. (2002), the marine myxobacterium *Nannocystis* sp. NU-2 produced a new kind of flocculating substance (NU-2 flocculant) com-

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posed of proteins and polysaccharides, and in another report (He et al., 2010), a novel bioflocculant known as HBF-3 was produced by a mutant of deep sea bacterium *Halomonas* sp. V3a.

The marine environments have been reported as good sources of interesting bacteria (Zhang et al., 2002), especially as a result of their unique environmental conditions, such as high pressure, low temperature and low nutrient concentrations. These characteristics influence the diversity of microorganisms whose morphological, physiological and metabolic adaptations are aligned towards survival therein as opposed to those of terrestrial microorganisms. It is thus anticipated that the marine environments could be a good reservoir of unique organisms producing unique bioactive compounds including bioflocculants that could be of relevance in various industrial processes (He et al., 2010).

Bioflocculants are environmentally safe, non-toxic and biodegradable, hence have a potential to alternately substitute synthetic flocculants currently utilized in water and wastewater treatment and various industries for solid-liquid separation (Lui et al., 2010). Furthermore, a number of studies have demonstrated the efficiencies of bioflocculants in removal of suspended solids, latex particles, microorganisms, chemical oxygen demand (COD), humic acids, heavy metals from waste streams, separation of oil from oil-water emulsions and fine coal processes (Ma et al., 2008; Zemouri et al., 2011). Such bioflocculants are polymers produced by microorganisms during their growth, with their flocculating activity being dependent on the characteristics of the flocculants (Gao et al., 2006).

Bioflocculants secreted by microorganisms (bacteria, algae, fungi and yeast) are extracellular biopolymeric compounds composed of proteins, glycoproteins, polysaccharides, lipids and glycolipids (Mabinya et al., 2011; Piyo et al., 2011). They have received considerable attention due to limitations of synthetic flocculants such as carcinogenicity and neurotoxicity (Zaki et al., 2011). Based on physico-chemical and rheological properties with noble functionality, these extracellular polymeric substances act as new biomaterials and some (polysaccharides) are found to contribute to various physiological activities in human beings including as anti-tumor, anti-viral, anti-inflammatory agents, and can act as inducers for interferon, platelet aggregation inhibitor and colony stimulating factor syntheses (Kumar et al., 2004). The need for new biodegradable bioflocculants with strong flocculating activity becomes imperative.

The genus *Oceanobacillus* was first proposed with the single specie, *Oceanobacillus iheyensis*. Currently, the genus is comprised of six species (Kim et al., 2007; Nam et al., 2008) which are alkaliphilic or halophilic microorganisms and very important in basic research as well as in industrial applications as they offer a multitude of actual or potential applications in various fields of biotechnology. For example, they play a crucial role in

food biotechnology for the production of fermented food and food supplements (Margesin and Schiner, 2001).

They have also been implicated in the production of bacteriorhodopsin, biopolymers for use in microbially enhanced oil recovery, and also compatible solutes and enzymes (Choudhry et al., 2003) in several industrial processes. In this work, we report on the potential of *Oceanobacillus* species for the production of bioflocculant that could be of relevance to water treatment in line with addressing food and water security in the Eastern Cape of South Africa.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The test bacteria was isolated from bottom sediment samples of Algoa Bay in South Africa as part of the culture collections of Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare, South Africa and maintained in 20% glycerol at -80°C. The bacteria were screened for bioflocculant production. The screening medium and cultivation in this study was as follows: 20 g of glucose, 1.0 g of peptone, 0.3 g of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 5 g of K_2HPO_4 and 0.2 g of KH_2PO_4 in 1 L of filtered natural sea water (Xia et al., 2008). The initial pH was adjusted to 7.0 by NaOH (0.1M) and HCl (0.1M). Two loop-full of bacterial colonies were inoculated in 50 ml of the cultivation medium and incubated with shaking at 160 rpm for 3 days at 30°C (Zhang et al., 2007). At the end of incubation period, 2 ml of the fermentation broth was centrifuged (4000 x g, 30 min) to separate the cells. The cell free supernatant was analyzed for flocculating activity. Bioflocculant production medium after optimization was as follows: 20 g of sodium carbonate, 1.3 g of tryptone, 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g of K_2HPO_4 and 0.2 g of KH_2PO_4 , in 1 L of filtered natural sea water. The initial pH was 10.08.

Identification of the bioflocculant-producing microorganism

DNA extraction was conducted via the boiling method whereby two to three colonies were suspended in 70 µl of sterile double distilled water. The samples were heated in a water bath at 100°C for 10 min, allowed to cool for 5 min and thereafter centrifuged at 3000 rpm for 5 min. The supernatant was transferred to a clean tube and stored at 4°C. This serves as the template in the PCR assay. PCR was carried out in 50 µl reaction volume containing 2 mM MgCl_2 , 2 U Supertherm Taq polymerase, 150 mM of each dNTP, 0.5 mM of each primer (F1: 59-AGAGTTTGATCITGGCTCAG-39; I = inosine and primer R5: 59-ACGGITACCTTGTACGACTT-39) and 2 µl template DNA. Primer F1 and R5 binds to base positions 7 to 26 and 1496 to 1476 of the 16S rRNA gene of *Streptomyces ambifaciens* ATCC 23877, respectively (Cook and Meyers, 2003). The primers in this study were used to amplify nearly full-length 16S rDNA sequences. The PCR programme used was an initial denaturation (96°C for 2 min), 30 cycles of denaturation (96°C for 45 s), annealing (56°C for 30 s) and extension (72°C for 2 min), and a final extension (72°C for 5 min). Gel electrophoresis of PCR products were conducted on 1% agarose gels to confirm that a fragment of the correct size had been amplified. Automated sequencing of the 16S rRNA genes of the bacterial isolate was performed using the Spectrumedix SCE2410 genetic analysis system with 24 capillaries. The sequencing reactions were performed according to the manufacturer's instructions, using the Big Dye version 3.1 dye terminator cycle sequencing kit (Applied

Biosystems) and 27F primer. The sequence was edited manually based on the chromatogram readings and used in the BLAST search to determine the most similar sequences.

Measurement of flocculating activity

The flocculating activity was determined using the method previously described by Kurane et al. (1994), in which kaolin clay was chosen as the suspended solid. 2 ml of the culture supernatant and 3 ml of 1% CaCl_2 were added into 100 ml of kaolin clay suspension (4 g/L) in 150 mL flask, gently shaken and left to stand still for 5 min. The control was prepared following the same procedure but the bioflocculant was replaced by fresh production medium. The absorbances of the upper phase were measured at 550 nm using spectrophotometer. The flocculating activity was expressed as flocculating percentage calculated as follows (Kurane et al., 1994):

$$\text{Flocculating activity} = [(A - B)/A] \times 100\%$$

Where, A is the optical density of the control at 550 nm and B is the optical density of the sample at 550 nm.

The effect of carbon, nitrogen sources and pH on bioflocculant production

It has been well documented that changing the carbon and nitrogen sources highly influenced bacterial growth and production of bioflocculant (Sheng et al., 2006). Hence, we assessed the effects of different carbon and nitrogen sources on bioflocculant production in the test bacterium. Carbon source candidates included glucose, sucrose, fructose, maltose, xylose, sodium acetate, phylate and sodium carbonate, while the nitrogen source candidates included ammonium sulphate, ammonium chloride (inorganic nitrogen sources) and casein, tryptone, yeast and urea (organic nitrogen sources) replacing peptone. To assess the effect of initial pH of the medium on bioflocculant activity, the pH of the medium was adjusted using HCl and NaOH in the pH range of 3 to 11 (Piyo et al., 2011). The mean values were used to represent the effect of carbon, nitrogen sources and initial pH of the medium and with respect to flocculating activity of the bioflocculant.

Effects of various cations

Studying the effects of cations, flocculant tests was conducted using the procedure elaborated above, however the CaCl_2 solution was replaced by various metal salt solutions, and the flocculating activity was measured. Solutions of KCl, NaCl, LiCl (monovalent), MnCl_2 , MgCl_2 (divalent), AlCl_3 and FeCl_3 (trivalent) were used as salt sources.

Time course of bioflocculant production

The composition of culture medium for the time course experiment was as previously elucidated above (Zhang et al., 2007). This was cultured under optimal growth conditions. The time course assays follow after the description of Gao et al. (2006) with modification. Briefly, saline suspension of the bacteria was used as seed culture for inoculum preparation. Seed culture (1% v/v) was inoculated into 150 mL medium in 500 ml flasks (prepared in duplicates) on a rotatory shaker (160 rpm) at 30°C. Sample was drawn at appropriate time intervals (every 12 h) for a period of 96 h. 2 ml of culture broth was centrifuged at 4000 g for 30 min and the cell free supernatant was used to determine the flocculating activity. The pH

and optical density (OD 660 nm) of the broth samples was also measured.

Extraction and purification of bioflocculant

Purification and characterization of the bioflocculant was done following the methods described elsewhere (Chen et al., 2002; Mabinya et al., 2011). Briefly, after fermentation, the culture solution (1 L) was centrifuged at 4,600 rpm for 30 min to remove bacterial cells. 1 L of distilled water was added to the upper phase and centrifuged at 4,600 rpm for 15 min to remove insoluble substances. To the supernatant, 4 L of ethanol were added, and the mixed solution was stirred and left to stand at 4°C for 12 h. The precipitate was vacuum dried to obtain the crude bioflocculant. The crude product was directly dissolved in 100 ml of distilled water to yield a solution, to which 100 ml of the mixed solution of chloroform and *n*-butyl alcohol (5:2 v/v) was added. After stirring, the mixture was set aside for 12 h at room temperature (about 20°C). The upper phase was centrifuged at 4,600 rpm for 15 min, vacuum-dried and then re-dissolved in distilled water, then dialyzed against de-ionized water overnight then vacuum dried to obtain a purified bioflocculant.

Characterization of purified bioflocculant

Qualitative detection of protein and carbohydrate were done using the Folin-Lowry (Lowry et al., 1951) and phenol-sulphuric acid (Dubois et al., 1956) methods, respectively. The presence of uronic acid content was measured by carbazole method as described by Cesaretti et al. (2003). The purified bioflocculant was characterized by using Fourier transform infrared spectrophotometer (Perkin Elmer System 2000, England). The dried bioflocculant powder was ground with potassium bromide (KBr) and pressed into pellets for FTIR spectral measurement in the frequency range of 4000 to 400 cm^{-1} (Kumar et al., 2004).

For scanning electron microscope observations, few drops of bioflocculant powder were dropped and fixed on the iron stub. The fixed specimen was gold coated and examined with a JSM-6390LV scanning electron microscope (Japan).

Jar-test experiment was used to determine the optimum dose of the purified bioflocculant for the clarification of kaolin clay suspension (4 g/L) at neutral pH. Different concentrations of the purified bioflocculant ranging from 0.2 to 1 mg/ml were used.

Thermo-stability of purified bioflocculant solutions were treated at three different temperatures (50, 80 and 100°C) for 30 min. Sample was drawn at appropriate time intervals (5 min) and then analyzed for residual flocculating activity as also previously described by Gong et al. (2008).

Statistical analysis

A one-way analysis of variance (ANOVA) test (Minitab Student Release 12) was used for determination of statistically significant differences of the percentage bioflocculant activity among the treatment means. Microsoft excel office 2007 version was used to determine means and standard deviations.

RESULTS AND DISCUSSION

Strain screening and identification

Preliminary screening of the bacteria for bioflocculant

Table 1. Effect of carbon and nitrogen sources on biofloculant activity.

Carbon source	Flocculating activity (%)	Nitrogen source	Flocculating activity (%)
Sodium carbonate	95.5 ± 1.8 ^a	Tryptone	84.0 ± 1.6 ^a
Xylose	89.5 ± 2.4 ^b	Ammonium nitrate	82.1 ± 3.4 ^b
Maltose	87.7 ± 3.3 ^c	Peptone	78.3 ± 2.9 ^c
Fructose	85.3 ± 2.5 ^c	Yeast extract	72.8 ± 3.3 ^c
Galactose	84.7 ± 3.8 ^c	Ammonium sulphate	66.6 ± 1.8 ^d
Sucrose	83.8 ± 2.0 ^c	Urea	63.7 ± 4.2 ^d
Glucose	72.4 ± 1.2 ^d	Casein	53.8 ± 2.1 ^e
Phylate	56.9 ± 0.1 ^e	Ammonium chloride	36.0 ± 0.1 ^f
Sodium acetate	17.9 ± 1.3 ^f		

The results are represented as mean values of triplicates ± standard deviation. Percentage flocculating activity with different letters (a, b, c, d, e and f) are statistically different ($p < 0.05$).

production revealed it to be positive with high flocculating activity of about 88%, thus necessitating its selection for further studies. The bacterial colony is light cream in color, glistening, round, entire and about 0.5 mm diameter. Also, the bacterium was gram-positive, rod-shaped and motile. Amplification of its 16S rRNA gene yielded the expected amplicon size of approximately 1.5 kb. Analyses of the partial nucleotide sequence of the amplified product revealed the bacteria to have 98% similarity with that of *O. iheyensis* HTE831 and the sequence was deposited in GenBank as *Oceanobacillus* sp. Pinky with (accession number HQ537126).

Effect of carbon sources and nitrogen sources on biofloculant activity

Effect of various carbon sources on biofloculant production by the bacteria was investigated. Of all the carbon sources, sodium carbonate yielded the highest flocculating activity (95.5%) (Table 1). On the other hand, phylate and sodium acetate were the least preferred carbon sources and are the rare case. Although, literature search revealed that a number of biofloculant producing bacteria prefer organic carbon sources for flocculant production, our test bacteria however showed high flocculating activity when this inorganic carbon source was used.

When examining the effect of nitrogen sources, tryptone, ammonium nitrate, peptone and yeast extract appeared favorable for biofloculant production with tryptone (organic nitrogen source) yielding the highest flocculation activity of 85% (Table 1).

A number of studies have been documented emphasizing the importance of carbon and nitrogen sources in the production of biofloculant (Suh et al., 2011). However, carbon and nitrogen source requirement may differ with different strains. For example, *Bacillus licheniformis* X14 preferred sucrose, starch and ethanol as favorable carbon sources for the production of ZS-7

biofloculant, while ammonium chloride was effectively utilized as nitrogen source (Lee et al., 2001). In another study (Sheng et al., 2006), *Klebsiella* sp. preferred maltose and urea as carbon and nitrogen source. In this study, sodium carbonate and tryptone were most favorable for the biofloculant production, even though sodium carbonate is rarely reported as preferred carbon sources. However, Lui et al. (2008) also reported tryptone as preferred organic nitrogen sources amongst other nitrogen sources for the biofloculant production by *Chryseobacterium daeguense* W6.

Effect of initial pH of the medium on biofloculant activity

The effect of initial pH on biofloculant production by the test bacteria are represented in Figure 1. All pH levels examined (pH range of 3 to 11) supported biofloculant production however a dramatic decrease of flocculating activity at pH 11 was noted. Although the biofloculant was produced almost through the initial pH range of 3 to 10, the highest flocculating activity for the biofloculant production was achieved at pH 10 hence this was used for this study. The change in pH may alter the charge status of the produced biofloculant hence variation of flocculating activity. The initial pH of the medium is one of the factors well reported to influence the production and flocculating activity of the biofloculant. The initial pH determines the electric charge of cells and oxidation-reduction potential which may affect the absorption of nutrients and the enzymatic reaction (Salehizadeh and Shojaosadati, 2001). However, this may differ with different strains. As example, in biofloculant PG.a21 Ca, the flocculation efficiency is lower at pH lesser than 7, but exhibit maximum activity at exactly neutral pH but this flocculant's activity then decreases with the increase of pH after pH 7 (Pan, 2009). Also, in the case of strain TJ-1 (Xia et al., 2008) and *Halomonas* sp. OKOH (Mabinya et al., 2011), pH 7 was the optimum for the biofloculant

Table 2. Effect of cations on bioflocculant activity.

Cation	Flocculating activity (%)
Na ⁺	30.7±3.5
Li ⁺	32.9±4.2
K ⁺	63.6±5.1
Mg ²⁺	67.9±1.5
Mn ²⁺	71.8±5.8
Ca ²⁺	86.3±3.4
Al ³⁺	88.2±2.6
Fe ³⁺	31.4±5.1

production. In another study (Deng et al., 2003) *Aspergillus parasiticus* preferred acidic conditions for synthesis, secretion and production of bioflocculant in the culture medium. Similar to our present findings, higher pH (above 7) appears more favorable for ZS-7 bioflocculant production by *B. licheniformis* X14 (Li et al., 2009). *Rhodococcus erythropolis* preferred alkaline pH (8.0 to 9.5) for bioflocculant production (Kurane et al., 1994).

Effect of cations on bioflocculant activity

Cations play a vast role in bioflocculation. They stimulate flocculating activity by neutralization and stabilizing the residual negative charge of functional groups and thereby forming bridges between particles (Salehizadeh and Shojaosadati, 2001). Their role is therefore to enhance initial adsorption of flocculants on suspended particles by decreasing the negative charge on both the polymer and the particle (Wang et al., 2011). In the case of this study, calcium chloride and aluminium chloride were the most stimulating cations on flocculating activity (Table 2). Similarly, as mentioned, this may have resulted due to our bioflocculant containing protein hence rich in amino acids containing carboxyl groups which contribute to negative charges of the particles, hence the cations were able to neutralize and form bridges between the particles allowing an improved bioflocculation. More carboxylate groups on the bioflocculant served as binding sites for the cations (Li et al., 2007); the bioflocculant and kaolin clay could form solid complexes through these calcium and aluminium. Also, the addition of these cations to the suspension increased the floc size resulting in enhanced sedimentation. In like manner, the flocculating activity of *R. erythropolis* protein flocculant and *Alcaligenes cupidus* bioflocculants were enhanced by the addition of Ca²⁺ and Al³⁺. However, aluminium chloride is one of the controversial salts as a result of the possible impact it has on Alzheimer disease, that is, reports on epidemiological, neuropathological and biochemical studies show an association between the neurotoxicity of aluminium and the pathogenesis of Alzheimer's disease (Banks et al., 2006; Cosa et al., 2012). For this reason, calcium chloride

was opted for as stimulating cation for the present study. The flocculating ability of MBFF19 bioflocculant produced by *Bacillus* sp. F19 (Zheng et al., 2008) and our previous report on *Halomonas* sp. OKOH was enhanced by calcium (Mabinya et al., 2011). The addition of Fe³⁺, Na⁺ and Li⁺ appeared to be the least effective cations (Table 2).

Effect of inoculum size on bioflocculant activity

Some studies (Gancel and Novel, 1994; Gong et al., 2008) reported that inoculum size amongst various bacteria's physiological properties plays a substantial role in biological development. Furthermore, the inoculum size has a significant effect on cell growth product formation as confirmed by Jang et al. (2001). The findings of the present study revealed that the flocculating activity initially increased with the increase in inoculum size; however, 2% inoculum size (1 ml in 50 ml production medium) used gave the highest flocculating activity (88.6%). The obtained flocculating activities at 2% inoculum size were significantly higher ($p < 0.05$) than other inoculum sizes tested. Thus, the optimal inoculum size allowed the adaptation of strains to the production medium thereby promoting the production of bioflocculants (Li et al., 2009).

Time course of the bioflocculant

Based on the assessments above, the optimal culture conditions for the bioflocculant production by the bacteria included the production medium containing: 20 g sodium carbonate, 1.3 g tryptone, 5 g K₂HPO₄, 2 g KH₂PO₄, and 0.2 g MgSO₄·7H₂O; pH 10 in 1 L seawater; inoculum size of 2% (v/v) (from the pre-culture of the bacteria inoculated into the fresh autoclaved production medium); incubation conditions of 160 rpm agitation speed, temperature of 30°C, and duration of 48 h. With these, the time course of the bioflocculant production by the bacteria was studied. As shown in Figure 2, the flocculating activity was initially observed to increase rapidly with culture time attaining a peak activity of 96.6% within 36 h of cultivation and thereafter a slight decrease and fluctuation in flocculating activity was observed. The pH remained relatively constant throughout the cultivation period, however a slight decline of pH at maximum flocculation activity was noted. The change in pH confirms the utilization of the nutrients in the medium by the strain (Piyo et al., 2011), that is, production of organic acids or presence of organic acid component in bioflocculant being produced. On the other hand, when optical density was used as a measure of concentration of bacteria in suspension, the OD_{660nm} profile showed that the cells rapidly acclimatized as reflected in the resulting bioflocculant activity. The bioflocculant activity

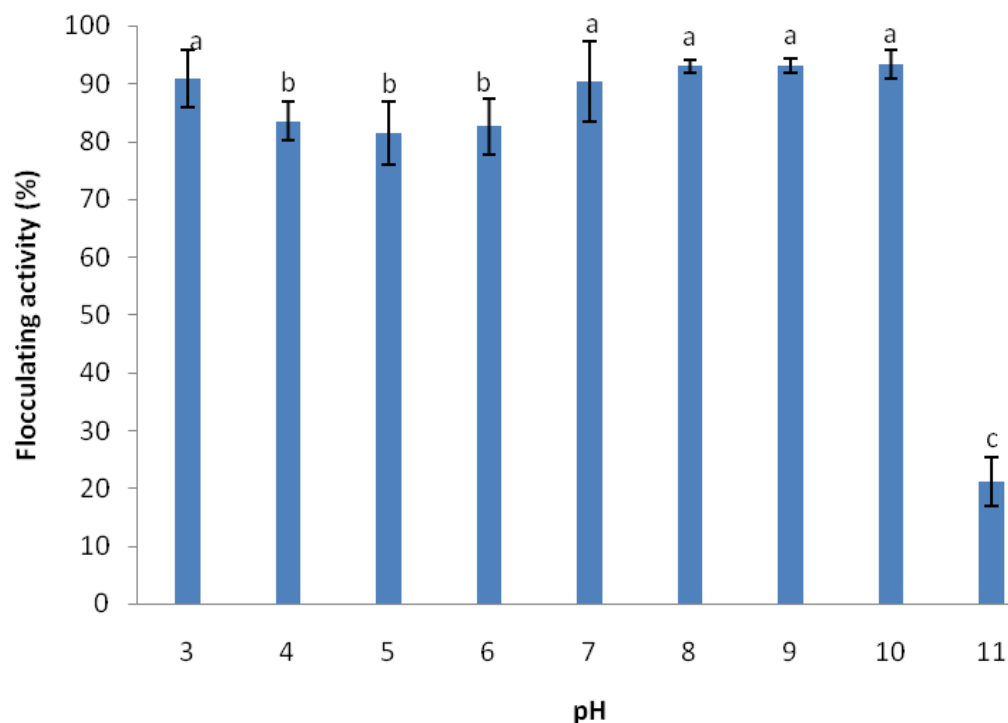


Figure 1. Effect of initial pH on bioflocculant activity by *Oceanobacillus* sp. Pinky. Error bars represent the standard deviation of mean value of triplicates. Percentage flocculating activity with different letters (a, b and c) are significantly ($p < 0.05$) different.

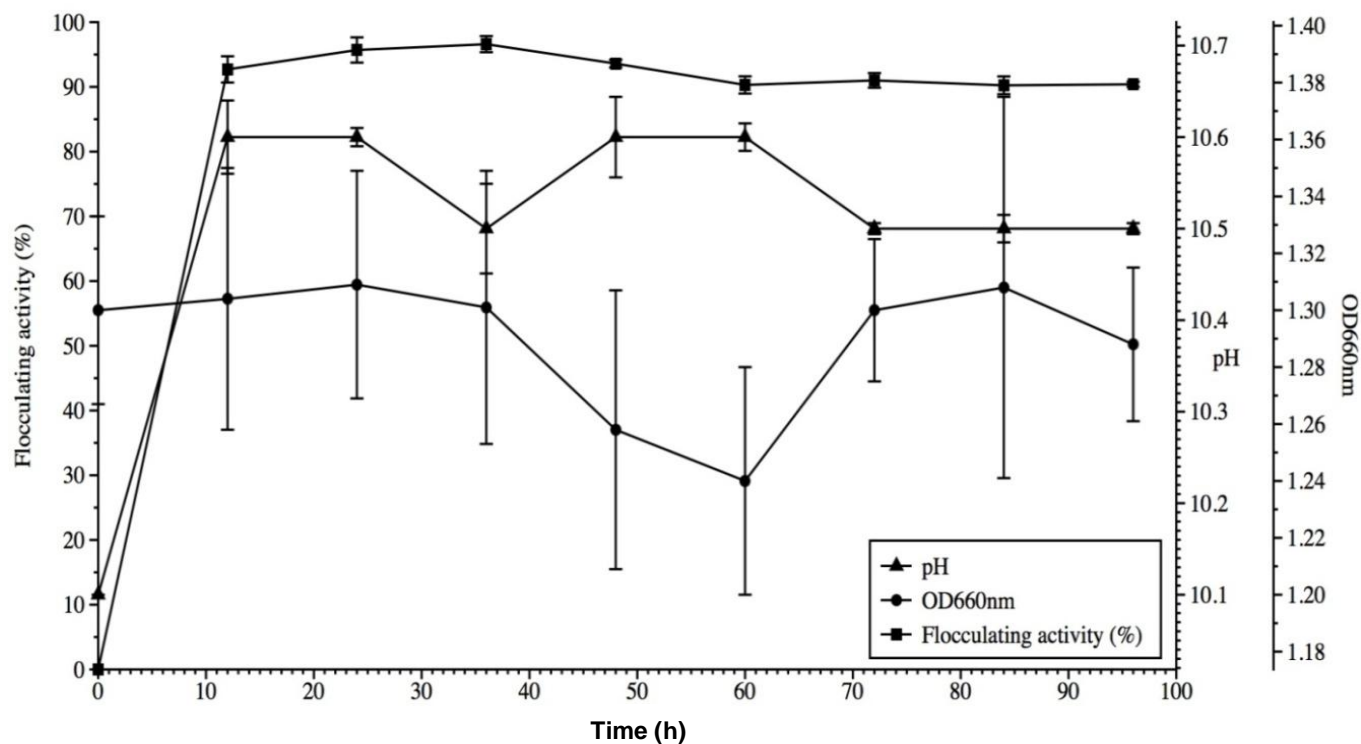


Figure 2. Time course of bioflocculant production by *Oceanobacillus* sp. Pinky. Error bars represent the standard deviation of mean value of triplicates

Table 3. Comparison of purified bioflocculant by *Oceanobacillus* sp. Pinky with other bioflocculants.

Strain	Bioflocculant yield (g/L)	Carbon/nitrogen sources in fermentation	Component	Reference
<i>Rhodococcus erythropolis</i>	0.205	n-Pentadecane, yeast extract, urea	Protein	Tadeka et al. (1991)
<i>Paecilomyces</i>	0.5	Glucose, yeast extract, casamino acid	Polysaccharide	Tadeka et al. 1985
<i>Proteus mirabilis</i> TJ-1	1.33	Glucose, peptone	Acid polysaccharide and protein	Xia et al. (2008)
<i>Bacillus</i> sp. Strain F19	1.47	Sucrose, yeast extract	Mainly polysaccharide	Zheng et al. (2008)
<i>Aeromonas</i> sp.	2.25	Sucrose, yeast extract, ammonium sulphate, urea		Li et al. (2007)
<i>Vagococcus</i> sp. strain W31	2.3	Glucose, ammonium sulphate	Polysaccharide	Gao et al. (2006)
<i>Oceanobacillus</i> sp. Pinky	2.44	Sodium carbonate, tryptone	Glycoprotein	Current study
Multiple-microorganism consortia MM1	15	Brewery wastewater, yeast extract, urea, ammonium sulphate	Glycoprotein	Zhang et al. (2007)

increased in proportion to the cell concentration increase and a high flocculating activity after 12 to 36 h of cultivation was observed. This may suggest that the bioflocculant was produced by biosynthesis (Gong et al., 2008). The slight decrease of flocculating activity could be empirical due to cell autolysis and enzymatic activity (Mabinya et al., 2011).

In other microorganisms, cultivation time for the release of flocculant substance into culture broth may differ. For example, in a study by Zheng et al. (2008), *Bacillus* sp. F19 produced a bioflocculant with flocculating activity increasing with the increase of cultivation time. However, the maximum flocculating activity was also reached after 36 h and then remained stable. In another study (Desouky et al., 2008), the flocculating activities of three isolates (QUST2, QUST6 and QUST9) belonging to *Bacillus* genus were observed to increase with increasing cultivation time. In contrast, our recent reports (Cosa et al., 2011; Piyo et al., 2011) showed that *Virgibacillus* sp. Rob and *Bacillus* sp. Gilbert produced their bioflocculants with maxima flocculating activity within the fourth day of cultivation. In the case of *Proteus*

mirabilis TJ-1 and *B. licheniformis* X14, maximum flocculating activity were achieved in 48 h, thereafter the activity decreased (Li et al., 2009; Xia et al., 2008).

Characterization of bioflocculant

The yield of the purified bioflocculant from *Oceanobacillus* sp. Pinky was 2.44 g/L (Table 3). This yield is relatively high as compared to those that could be found in literature as shown in Table 3. The surface morphology structure of the purified bioflocculant and its interaction with kaolin clay suspension was studied using scanning electron microscopy (SEM). The bioflocculant was whitish in color and crystal-linear like structure was observed. The picture of kaolin clay particles before suspension and flocculation revealed finely scattered particles; however when kaolin clay particles were flocculated by test bioflocculant, a link between these formed larger flocs enabling rapid sedimentation as a result of gravity during the process of flocculation. Our findings were similar to that reported by Xia et al. (2008). Quali-

tative analyses revealed the presence of protein (5.8%), neutral sugar (12.1%) and uronic acids (82.9%) in the final purified bioflocculant suggesting it to be a glycoprotein. Similar bioflocculants have been reported before and glycoprotein flocculants can be applied in various fields including biotechnology and nanotechnology (silicon wafers, lipid films and liposomes) (Lungmann et al., 2007).

Functional groups and elemental analysis

Some functional groups in the purified bioflocculant were examined using the FTIR spectrophotometry and the results are shown in Figure 3. A broad stretching peak at 3437.04 cm^{-1} was displayed from the spectrum indicative of the presence of hydroxyl and amino group. Aliphatic C-H bonds were observed as indicated by small peak at 2394.51 cm^{-1} . The peaks at 1648.49 and 1401.14 cm^{-1} suggested the presence of carboxyl groups with C=O in an amine group and an asymmetric C=O stretching in the carboxylate, respectively (Yim et al., 2007). The peak at 1235.02 cm^{-1}

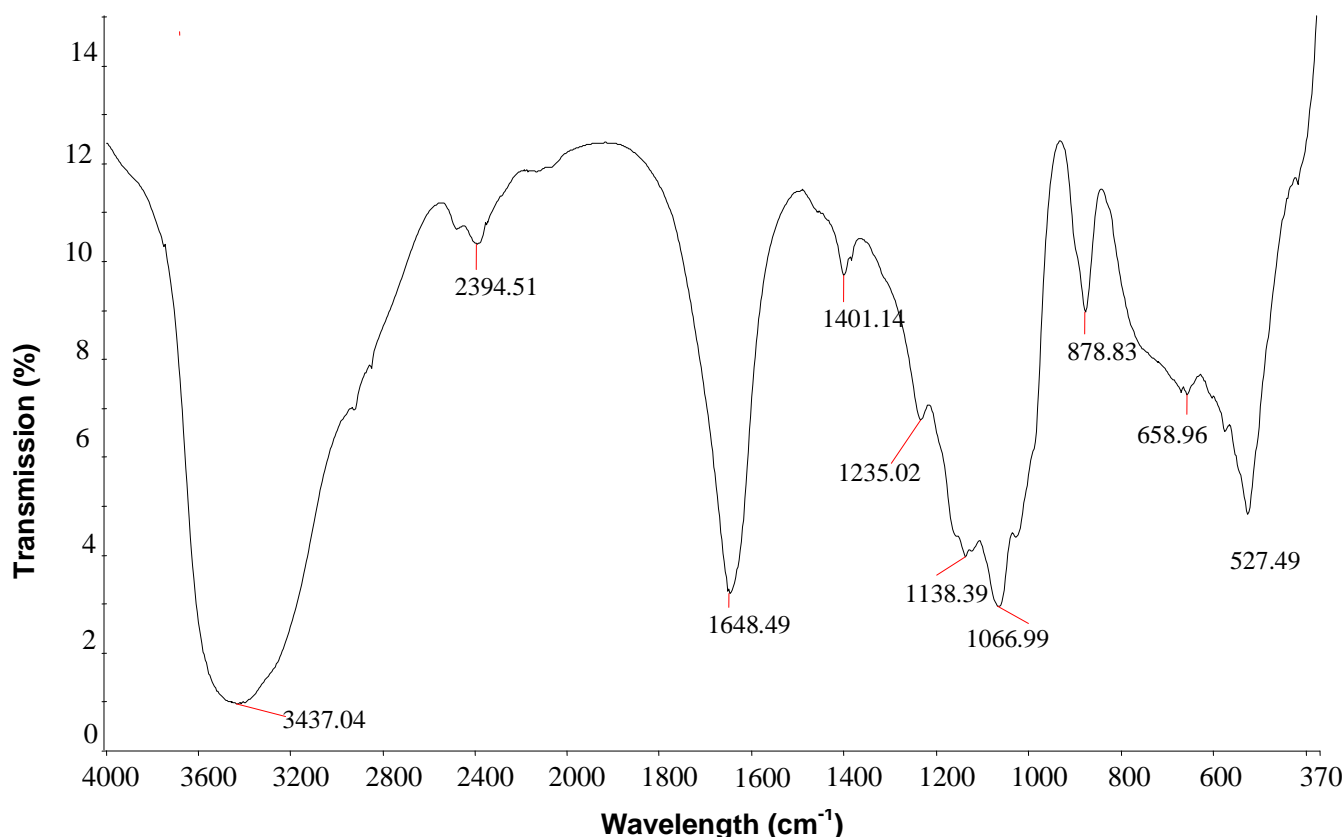


Figure 3. Infrared spectra of purified polysaccharide bioflocculant.

may be indicative of the C-O stretching in ether or alcohol, while the absorption peaks at 1066.99 cm^{-1} may also be attributed to the asymmetrical stretching vibration of a C-O-C ester linkage (Zheng et al., 2008). The small weak peaks at 878.83 to 527.49 cm^{-1} are characteristics of all sugar derivatives (Deng et al., 2003).

Consequently, these findings presented the presence of carboxyl, hydroxyl and amine groups on the bioflocculant, which are the favoured groups for the process of flocculation. These groups allow linkage with the H^+ and OH^- present on the surface of the particles forming hydrogen bonds when the bioflocculant chains approach the particles' surface (Zheng et al., 2008). Our findings corroborate with a number of reported studies (Feng et al., 2008; Wang et al., 2010; Xiong et al., 2010).

Further characterization of the bioflocculant with EDX elemental analysis revealed the presence of carbon, nitrogen, phosphorus and sulfur with 13.37, 6.63, 12.2 and 2.76%, respectively.

Sulfur was present as minor element on the surface of purified bioflocculant. The presence of these non-sugar components although they are usually in small amounts, they give flexibility and stabilize the bioflocculant (Bhaskar, 2003). Yim et al. (2007) reported that the elemental analysis of p-KG03(bioflocculant) proportions of

carbon, hydrogen, nitrogen and sulfur were 32.2, 3.9, 0.5 and 10.3%, respectively. These results further substantiate the FTIR analysis findings and hence confirming the presence of carboxyl and amino groups present in the bioflocculant.

Thermal stability of the bioflocculant

The thermal stability of the bioflocculant produced by our test bacteria was evaluated. The findings of this study show that the bioflocculant activity decreased by ~7% when the bioflocculant was heated at 50°C for 15 min, while after heating for 30 min, approximately 17% of the flocculating activity was lost. A fluctuation in flocculating activity was observed when the bioflocculant was heated to 80°C . After heating the bioflocculant at 100°C for 30 min, the flocculating activity was decreased by 16%. Hence, it was presumed that the bioflocculant was thermally stable. The slight decrease on flocculating activity may be due to partial denaturation of some of the protein component of the bioflocculant. A similar phenomenon was observed with *Serratia ficaria* (Gong et al., 2008). However, in contrast to our findings, their bioflocculant was predominantly described as polysaccharide.

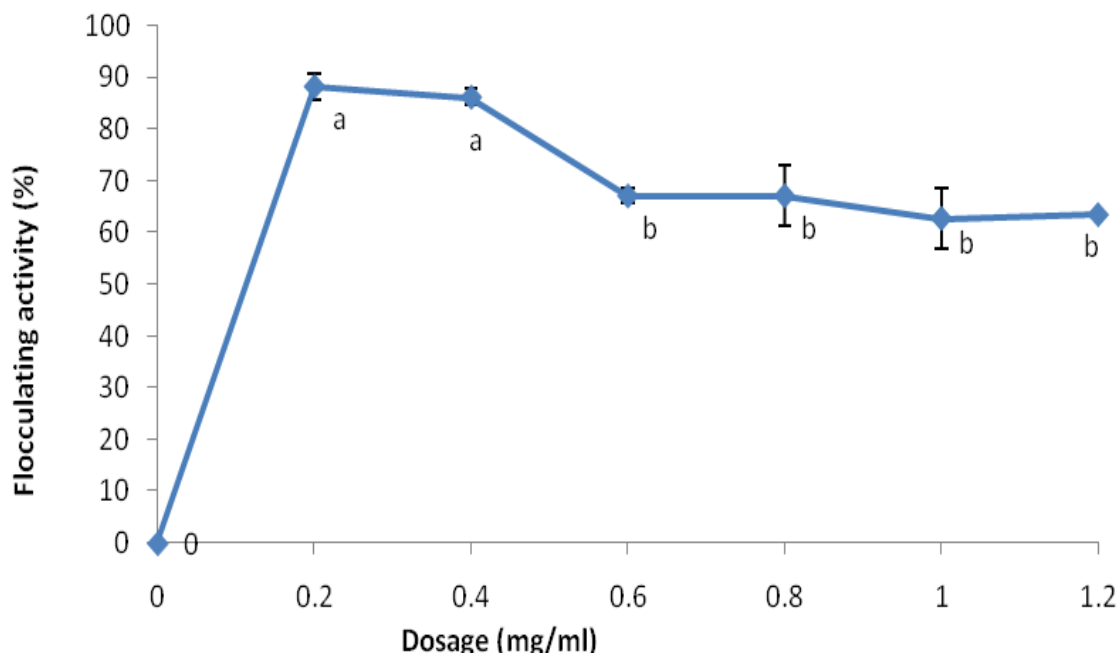


Figure 4. Effect of bioflocculant concentration on flocculation. Percentage flocculating activity with different letters (a, b) are significantly ($p < 0.05$) different.

Jar-test

The optimum dose of the purified bioflocculant required for the flocculation of kaolin suspension was determined using Jar test experiment, and results reveal the optimum dose to be 0.2 mg/mL resulting in flocculating activity of 88.2% ($p < 0.05$). When the bioflocculant dose was increased beyond this optimum, flocculating activity decreased (Figure 4). This decrease may be attributed to a phenomenon referred to as “flocculation deterioration” whereby some colloidal particles become enclosed up by the highly concentrated flocculant and a “colloid protection function” forms and consequently a decrease in flocculation activity is observed (Liang et al., 2011). Excessive bioflocculant would cover with the disperse matters, and is also blocked the formation of bigger flocs.

Effect of pH on flocculation

It has been reported that alterations of pH subsequently change the bioflocculant charge status and surface characteristics of suspended materials consequently changing the flocculating ability and flocculation (Ma et al., 2008). In this present study, the effect of pH on flocculating activity was examined using 0.2 mg/ml concentration and pH range of 3 to 11 and results are depicted in Figure 5. Our finding revealed that flocculation activity was observed throughout the studied pH range (3 to 11) however, the maxima activity (87.5%) was

obtained at neutral pH. Thereafter, the flocculating activity slightly varied at alkaline pH (9 to 11). At lower pH, the bioflocculant and the kaolin may probably adsorb the H^+ leading to reduced flocculation activity; on the other hand, at high pH, the OH^- ions may hinder the formed complex of the bioflocculant and kaolin particles in the mixture, however this mediating effect may be stronger at neutral pH. The subsequent variations in flocculating activity were as a result of change of pH affecting the charge status of the bioflocculant and that of the surface characteristics of suspended materials (Zhang et al., 1999). A similar phenomenon was reported by He et al. (2010) for bioflocculant produced by deep sea bacteria mutant *Halomonas* sp. V3a'. pH requirements for different reported bioflocculant(s) vary significantly. Prasertsan et al. (2006) reported *Enterobacter cloacae* WD7 bioflocculant with the optima pH for flocculating activity at pH 6.0. CBF-F26 bioflocculant was produced by mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaericus* F6, and high flocculation efficiency was achieved under neutral and weak alkaline conditions (Wang et al., 2010).

Comparative analysis of flocculation efficiencies of different chemical flocculants and the bioflocculant

The effectiveness of our test bioflocculants was compared with those of some conventional synthetic flocculants (polyacrilamide (PAM), polyethylimine, alum

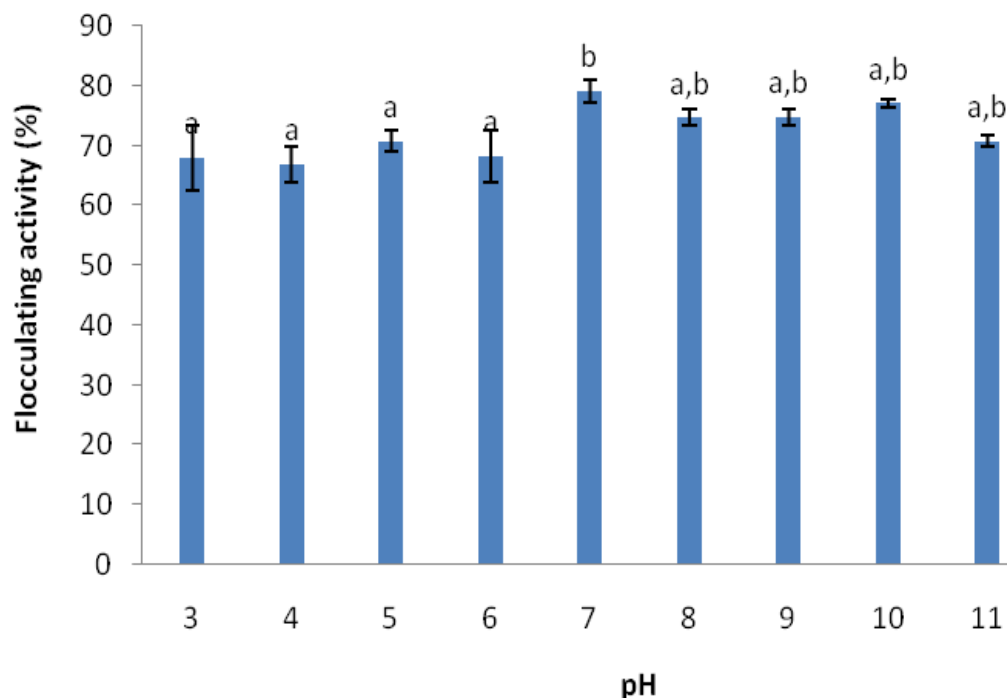


Figure 5. Effect of pH on flocculation. Percentage flocculating activity with different letters (a, b) are significantly ($p < 0.05$) different.

Table 4. Comparison of bioflocculant efficiency with chemical flocculants.

Flocculant	Dosage (mg/mL)	Rate of floc formation	Flocculating activity (%)
Polyacrylamide	0.1	+++	95.2±1.7 ^a
Polyethylimine	0.3	++	82.6±5.7 ^c
Alum	1	++	70.9±5.2 ^d
Ferric chloride	0.8	+++	74.5±7.3 ^d
Bioflocculant	0.2	+++	88.3±1.2 ^b

The results are represented as mean values of triplicates \pm standard deviation. Percentage flocculating activity (FA) with different letters (a, b, c, d) are significantly ($p < 0.05$) different. + = low; ++ = medium; +++ = high.

and ferric chloride) at a concentration range of 0.1 to 1 mg/ml and neutral pH for flocculating kaolin suspension under similar conditions and results are depicted in Table 4. The results show that polyacrylamide was most efficient, followed by our bioflocculant, as revealed by the optimum dose of 0.1 and 0.2 mg/ml as compared to 0.3, 0.8 and 1 mg/ml for polyethylimine, ferric chloride and alum, respectively. This result suggests that the purified bioflocculant could stand as alternative to chemical flocculants.

Conclusions

This study has confirmed the potentials of *Oceanobacillus*

sp. Pinky as a source of glycoprotein bioflocculant(s) that could stand as alternatives to hazardous inorganic and synthetic flocculants. The optimal bioflocculant production conditions included sodium carbonate and tryptone as carbon and nitrogen sources, respectively, an inoculum concentration of 2 % (v/v) as well as pH of 10. The bioflocculant was heat stable at the low optimum dose of 0.2 mg/mL of the purified bioflocculant for the flocculation of kaolin clay; the neutral pH required for optimal activity; and its similar flocculating efficiency in comparison with polyacrylamide suggests that it could stand as alternative to chemical flocculants. It is suggested that further development of process condition for large scale production and applicability in the treatment of various types of wastewater be pursued and this is a subject of further

investigation in our group.

ACKNOWLEDGEMENTS

We thank the University of Fort Hare for funding this research. The first author is thankful to the National Research Foundation (NRF) of South Africa for a study bursary. The NRF support is also acknowledged for the Risk and Vulnerability Assessment Centre at the University of Fort Hare.

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